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Amplification of the *HER2* gene occurs in more than 20% of breast cancers and is associated with aggressive tumor growth. Additional genetic mechanisms including translational deregulation may contribute to overexpression of the HER2 oncoprotein. The HER2 mRNA contains a conserved short upstream open reading frame that represses downstream translation in mammalian cells, in cell free extracts and in *S. cerevisiae*. The inhibitory effect of the upstream open reading frame does not depend on the precise 5' end of the mRNA, the uORF coding sequences or the nature of the downstream cistron. Rather, the naturally short intercistronic spacing between the upstream open reading frame and the HER2 coding region is critical for its inhibitory effect. Ribosomes that have translated the upstream open reading frame are able to reinitiate only inefficiently at the HER2 initiation codon but may reinitiate further downstream. These studies demonstrate that the uORF has a major repressive impact on HER2 protein expression.

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Robert P. Gladu 12/22/98

PI - Signature Date

4. Table of Contents

5. Introduction.

Overexpression of the HER2 oncoprotein occurs in 20-30% of breast tumor cells and is predictive of aggressive tumor growth (8, 13, 15). As well, *HER2* functions as an oncogene in cell culture transformation assays (4, 5, 11). Thus, *HER2* may be a causal factor in the development and/or progression of breast cancer. The importance of *HER2* overexpression in breast cancer is highlighted by the recent development and FDA approval of a humanized monoclonal anti-HER2 antibody for treatment of breast cancer.

The mechanism by which the HER2 protein is overexpressed in tumor cells is only partially understood. Several studies have highlighted a correlation between with *HER2* gene amplification and HER2 protein overexpression [reviewed in (8)]. These observations suggest that the abnormally high *HER2* gene copy number in breast cancer cells may account for its overexpression. However, in some tumor cells, transcriptional activation of *HER2* occurs without detectable gene amplification (10). Moreover, as our work suggests, translational controls also contribute to the overexpression of the HER2 protein in tumor cells (1).

In most eukaryotic mRNAs, the 5' proximal AUG codon serves as the site for initiation of protein synthesis. However, a large number of mRNAs encoding growth factors and cellular receptors, including a majority of oncogene transcripts, contain AUG codons and associated short open reading frames (uORFs) upstream from the major translational start site (9). The role of these uORFs is largely uncharacterized though an increasing number of examples illustrate the potential for these elements to influence expression of the downstream reading frame [reviewed in (6)].

The HER2 mRNA contains an uORF that is highly conserved among mammalian species and is preserved in at least the few cell lines derived from breast tumor that have been studied (1). The uORF inhibits translation of a downstream reporter gene in mammalian cell transfection assays (1). This research project aimed to elucidate the role of this uORF in breast cancer by (i) determining the mechanism of the uORF inhibitory effect using *S. cerevisiae* to complement studies in mammalian cells and by (ii) by ascertaining the functional consequences of *HER2* uORF-mediated inhibition for cellular growth control.

6. Body.

Mechanism of inhibitory effect of the uORF.

These studies originally utilized *S. cerevisiae* as a model system to investigate the regulatory mechanism by which the *HER2* uORF inhibits downstream translation. Studies in the first year of this grant revealed that the position of the uORF within the transcript leader appeared to be critical for its inhibitory effect in yeast. However, several difficulties were encountered with these experiments. For example, variation among replicate yeast transformants was quite large. Also, evidence from other studies cast doubt on the hypothesis that uORF-mediated regulatory mechanisms are identical in yeast as in mammalian cells. In particular, another uORF that acts in a peptide sequence dependent manner in mammalian cells, acts in a peptide-independent manner in yeast [(3) and data not shown].

Because of these uncertainties about the reliability of the yeast system, subsequent investigations of the function of the uORF were conducted primarily in mammalian cells. Nucleic acid databases contain discrepancies regarding the 5' end of the HER2 mRNA. Therefore, we measured the translational effects of the uORF, when it was contained in the 178 nt transcript leader that we and others have detected or when it was in a leader consisting of only the highly conserved 98 nt 3'-most portion (1, 2, 7, 14, 16). The uORF repressed downstream translation ~5-10 fold in both leaders (data not shown). Next, we tested the importance of the conserved coding sequences of the uORF. Mutants having substitutions affecting predominantly the coding sequences or affecting both the coding and nucleotide sequences of the uORF retained the inhibitory effect (Fig. 1). These studies indicated that the inhibitory effect of the *HER2* uORF in mammalian cells does not depend on the precise 5' end of the mRNA or on the coding content of the uORF.

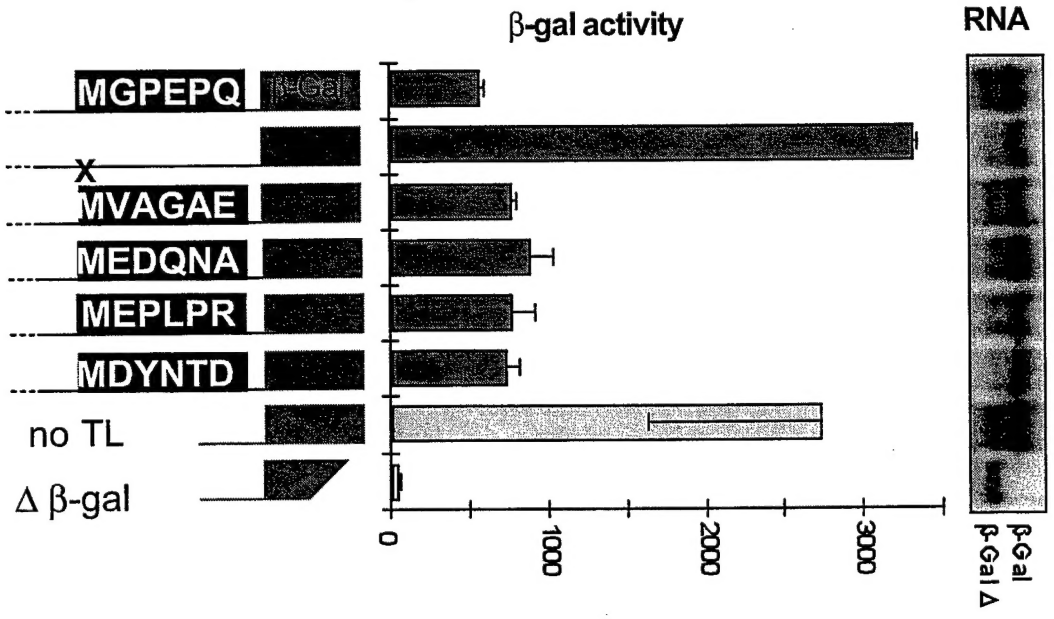

Figure 1. The uORF inhibits downstream translation by a sequence-independent mechanism. The wild-type uORF (MGPEPQ) and indicated missense mutants and controls having no transcript leader (no TL) or a truncated, enzymatically inactive ß-gal (Δ ß-gal) were transfected into COS7 cells and ß-gal activities were measured. All constructs expressed similar levels of ß-gal RNA (right), consistent with observed differences being due to a translational mechanism.

Guided by our previous results in *S. cerevisiae*, we next prepared constructs in which various intercistronic spacers were inserted between the uORF and the downstream cistron. Extension of the intercistronic spacing to >~50 nucleotides resulted in an increase in downstream translation. Moreover, spacer insertion reduced expression for each corresponding AUG$^-$ control. Thus, the overall effect of the uORF, measured as the ratio of expression from the corresponding constructs lacking the upstream AUG to

those containing it, was nearly eliminated by extending the intercistronic spacing (Table 1).

Spacer	Length (nt)	uORF effect (AAG/AUG)
wild-type	5	6.9
UL4	10	5.0
	50	2.4
	116	1.7
	151	1.4
GCN4	172	1.7
HER2 (5' end)	91	0.4

Table 1. Effects of inserting intercistronic spacer sequences inserted between the uORF and the ß-gal AUG codon. The uORF effect refers to ß-gal expression from a plasmid containing a point mutation in the upstream AUG codon (to AAG) divided by expression from the corresponding construct containing the upstream AUG codon. The uORF effect decreases from ~7 with the wild-type spacing to < 2-fold with ≥116 nt spacing.

These results suggest the ribosomes that have translated the uORF can reinitiate, but only after having resumed scanning for a relatively long distance. In the natural *HER2* mRNA, ribosomes that translate the uORF might therefore be unable to reinitiate at the *HER2* AUG codon but rather reinitiate further downstream. The next two AUG codon that ribosomes would encounter are surrounded by moderately good context of nucleotides for translation initiation (GACaugA and GACaugC) and are in the HER2 reading frame (Fig 2)

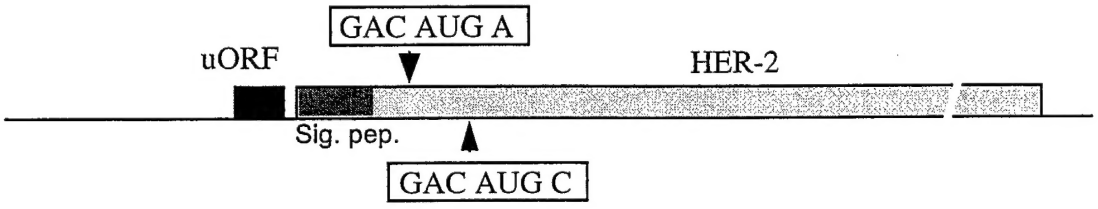

Figure 2. Structure of the *HER2* mRNA showing the positions of the 3rd and 4th AUG codons that are downstream from the signal peptide (Sig. pep.) and may serve as initiation sites for synthesis of a N-terminally truncated HER-2 protein.

To test whether the uORF influences ribosomal reinitiation at these downstream sites, we prepared chimeric plasmids in which the ß-gal initiation codon was positioned 91 nucleotides downstream from the uORF termination codon. Therefore, ribosomes reinitiating at the ß-gal AUG codon would have had to traverse the natural 5' end of the *HER2* cistron. In transfection experiments, the uORF paradoxically increased ß-gal expression (Table 1). These results suggest that the uORF may serve to facilitate ribosomal reinitiation at a downstream AUG codon thereby producing a N-terminally

truncated HER2 protein. Interestingly, others have shown that N-terminal truncated HER2 is more potent as a transforming gene than is the full length protein (12), although the truncation was much larger in those experiments than in the protein predicted by our results. In preliminary experiments, we have detected a faint band co-migrating with the putative truncated HER2 protein. A future goal is to determine whether this band in fact represent the N-terminally truncated protein and if so, to evaluate its transforming potential and determine whether its expression is increased in other cell types or growth conditions.

The observed effects of the uORF on downstream translation suggested, but only indirectly, that the uORF is actually translated. To evaluate this prediction more directly, we constructed in-frame fusions of the HER2 uORF to lacZ such that ß-gal protein synthesis depends on ribosomes translating the uORF. As shown in Fig. 2, transfection assays confirmed that the uORF is translated. We raised antiserum to uORF peptide for use in future studies investigating expression of the uORF-encoded peptide in cells.

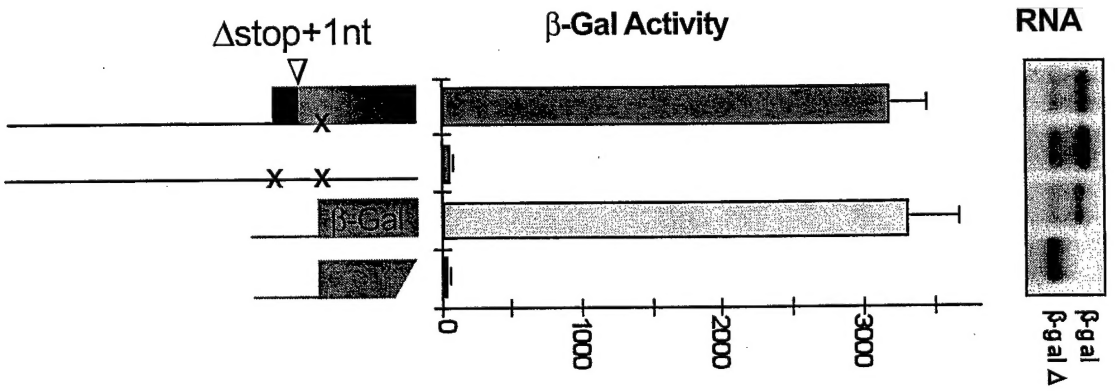

Figure 3. Translation of the uORF. An in-frame fusion of the uORF to *lacZ* was constructed by deleting the uORF stop codon and inserting one nucleotide. This plasmid and the indicated controls were transfected into COS7 cells and ß-gal activity and RNA levels were measured. These results demonstrate translation initiation at the uORF AUG codon and strongly suggest that the uORF is indeed translated.

Functional consequences of inhibition by the uORF.

uORF affects HER2 protein expression.

The above studies revealed that the uORF inhibited downstream translation of a reporter gene. To test whether the uORF similarly affected expression of the authentic HER2 protein, we placed the wild-type HER2 cistron with a FLAG epitope tag downstream from its own transcript leader in place of *lacZ*. Transfection of the uORF-containing plasmid compared to an upstream AUG$^-$ mutant confirmed that the uORF repressed expression of the HER2 protein similar to its effect on the heterologous ß-gal reporter gene (Fig. 4). Confirmatory results were obtained in examining cells transfected with HER2 constructs lacking the FLAG tag and probing with anti-HER2 antisera and in

tunicamycin treated cells (data not shown). Thus, the uORF represses the abundance of the authentic HER2 protein.

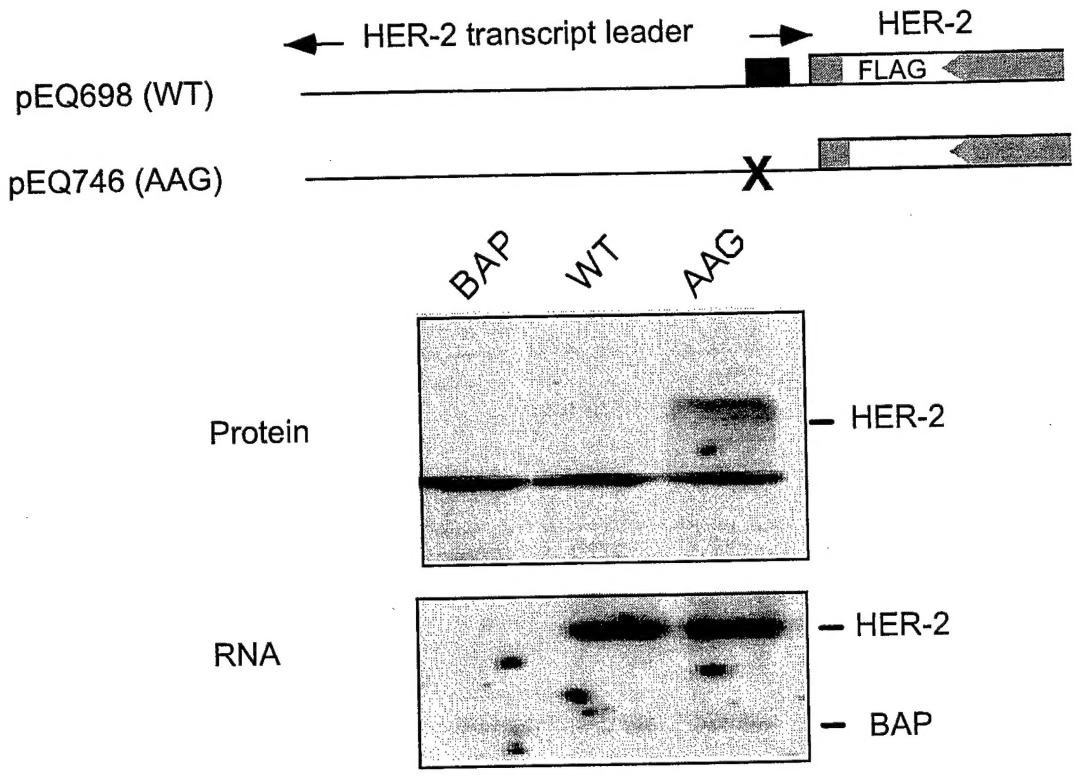

Figure 4. Repression of FLAG-tagged HER2 protein expression by the uORF. The plasmids depicted at the top were transfected into COS7 cells along with a control FLAG construct (BAP). Cell extracts were analyzed by immunoblot for FLAG-containing HER2 protein and RNA purified from transfected cells was detected with a FLAG epitope-specific oligonucleotide probe.

We attempted to assess whether the uORF affects HER2 transforming activity as a result of its inhibitory affect on HER2 protein expression. We hypothesized that either two mechanisms might lead to disproportionate transforming activity of constructs containing the uORF despite the uORF's inhibitory effect on downstream translation. First, as described above, the uORF might facilitate expression of a N-terminally truncated HER2 protein with greater transforming activity than the full length protein. Secondly, the uORF may limit ribosomal density on the HER2 mRNA and thereby enable some cotranslational process, such as folding, to occur in a more optimal manner such that HER2 protein may be more active as a result of the uORF.

Our attempts to transform cells with *HER2* have been unsuccessful, suggesting that *HER2* is not a very potent transforming gene. We recently obtained the mutant version of the rat *neu* gene that contains an activating mutation in the transmembrane domain. We are in the process of constructing plasmids having this mutation which should be more active in transformation and with which we should be better able to evaluate the impact of the uORF. Finally, we attempted to establish stable cell lines to

detect subtle effects of *HER2* on cellular growth. However, we encountered technical obstacles in these experiments and have been unable to establish cell lines even with control vectors having only the antibiotic resistance gene (neo or puro) without the *HER2* gene. Trouble-shooting of this technical obstacle is continuing. Thus, we do not yet know whether the uORF affects the activity of HER2 protein in proportion to its effect on protein abundance.

7. Conclusions.

These studies have shown that two distinct translation mechanisms affect HER-2 protein expression. One of these is cell type-specific, resulting in inefficient translation of HER2 in primary but not transformed cells. The second occurs in all cells tested and is mediated by an uORF. Although the *cis*-acting sequences responsible for the cell type differences is unknown, the full-length, FLAG-tagged plasmids constructed here will be useful for delineating these sequences.

The HER2 uORF inhibits expression in all mammalian cell types tested, in *S. cerevisiae* and in cell free translation extracts. The inhibitory effect of the uORF does not depend on the precise 5' end of the mRNA, the coding content of the uORF, or the nature of the downstream cistron. However, the intercistronic spacing between the uORF and the downstream cistron is critical. Ribosomes translate the uORF but then fail to reinitiate efficiently at the HER2 AUG codon because of its proximity to the termination codon of the uORF. Some ribosomes do reinitiate at a downstream AUG codon, possibly producing a truncated HER2 protein. Establishing the existence and function of this protein is under investigation. We do not yet know whether the uORF affects the activity of HER2 protein in proportion to its effect on protein abundance. However, this research project has clearly established that the uORF has a profound impact on *HER2* gene expression.

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9. Appendicies.

none

10. Publications & Personnel.

Manuscripts

1. Child, S.J., M.K. Miller and A.P. Geballe. Cell type-dependent and -independent control of HER-2/*neu* translation. 1999. Int. J. Biochem. and Cell Biol. *in press.*

2. Child, S.J., M.K. Miller and A.P. Geballe. Functional analysis of the HER-2/*neu* upsteam open reading frame. in preparation.

Abstracts

1. Child, S.J., Miller, M.K. and Geballe, A.P. Translational Regulation of HER2/neu expression in primary and breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, October 31 – November 4, 1997, Washington, DC.

2. Child, S.J., Miller, M.K. and Geballe, A.P. Translational regulation of HER2 expression. Translation and Stability of mRNA, October 12-14, 1997, San Francisco.

3. Child, S.J., Miller, M.K. and Geballe, A.P. Functional analysis of the HER2 uORF. Translational Control meeting at Cold Spring Harbor, September 9-13, 1998

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